Cytochrome *c* oxidase contains an extra charged amino acid cluster in a new type of respiratory chain in the amino-acid-producing Gram-positive bacterium *Corynebacterium glutamicum*

Junshi Sakamoto,[1] Takatsugu Shibata,[1] Tadashi Mine,[1] Ryoko Miyahara,[1] Tomokimi Torigoe,[1] Shunsuke Noguchi,[1] Kazunobu Matsushita[2] and Nobuhito Sone[1]

Author for correspondence: Junshi Sakamoto. Tel: +81 948 297823. Fax: +81 948 297801. e-mail: sakamoto@bse.kyutech.ac.jp

[1] Department of Biochemical Engineering and Science, Kyushu Institute of Technology, Kawazu 680-4, Iizuka, Fukuoka-ken 820-8502, Japan

[2] Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Yamaguchi 753-0841, Japan

The membranes from *Corynebacterium glutamicum* cells contain a hydrophobic di-haem C protein as the cytochrome *c* subunit of the new type of cytochrome *bc* complex (complex III in the respiratory chain) encoded by the *qcrCAB* operon [Sone, N., Nagata, K., Kojima, H., Tajima, J., Kodera, Y., Kanamaru, T., Noguchi, S. & Sakamoto, J. (2001). *Biochim Biophys Acta* 1503, 279–290]. To characterize complex IV, cytochrome *c* oxidase and its structural genes were isolated. The oxidase is of the cytochrome aa_3 type, but mass spectrometry indicated that the haem is haem As, which contains a geranylgeranyl side-chain instead of a farnesyl group. The enzyme is a SoxM-type haem–copper oxidase composed of three subunits. Edman degradation and mass spectrometry suggested that the N-terminal signal sequence of subunit II is cleaved and that the new N-terminal cysteine residue is diacylglycerated, while neither subunit I nor subunit III is significantly modified. The genes for subunits II (*ctaC*) and III (*ctaE*) are located upstream of the *qcrCAB* operon, while that for subunit I (*ctaD*) is located separately. The oxidase showed low enzyme activity with extrinsic substrates such as cytochromes *c* from horse heart or yeast, and has the Cu_A-binding motif in its subunit II. A prominent structural feature is the insertion of an extra charged amino acid cluster between the β2 and β4 strands in the substrate-binding domain of subunit II. The β2–β4 loop of this oxidase is about 30 residues longer than that of major cytochrome *c* oxidases from mitochondria and proteobacteria, and is rich in both acidic and basic residues. These findings suggest that the extra charged cluster may play a role in the interaction of the oxidase with the cytochrome *c* subunit of the new type of *bc* complex.

Keywords: cytochrome aa_3, dihaem cytochrome *c*, glutamate fermentation, high-G+C Gram-positive bacteria

INTRODUCTION

Cytochrome *c* is a peripheral membrane protein located in the periplasmic space of bacteria as well as in the mitochondrial intermembrane space of eukaryotes, which transfers electrons from the cytochrome bc_1 complex (complex III) to cytochrome *c* oxidase (complex IV) in the respiratory chain. Gram-positive bacteria have no outer membrane or periplasmic space and contain four types of cytochrome *c* with a membrane anchor. Cytochrome *c*-551 in the thermophilic *Bacillus* species is bound to the cell membrane by a diacylglycerol moiety covalently linked to the N-terminal cysteine residue (Noguchi *et al.*, 1994), whereas cytochrome

Abbreviations: DG, *n*-decyl-D-glucoside; MALDI, matrix-assisted laser desorption ionization; MEGA 9, *n*-nonanoyl *N*-metylglucamide; MEGA 10, *n*-decanoyl *N*-methylglucamide; PTH, phenylthiohydantoin; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AB052748 and AB052749.

c-550 in *Bacillus subtilis* is bound to the membrane by its N-terminal hydrophobic signal peptide (von Wachenfeldt & Hederstedt, 1993). The third type, cytochrome caa_3-type oxidase, contains a cytochrome *c* moiety genetically fused to the C-terminus of subunit II and accepts electrons directly from the cytochrome *bc* complex in the absence of another cytochrome *c* (Sone *et al.*, 1987). Finally, we have recently identified a new type of cytochrome *c* which is genetically fused to the cytochrome *c* subunit of the cytochrome *bc* complex from *Corynebacterium glutamicum* (Sone *et al.*, 2001). *C. glutamicum* is an aerobic high-G+C Gram-positive bacterium of industrial importance in the production of amino acids used as nutritious additives in food and fodder. Electrophoretic analysis indicated that the organism has only one *c*-type cytochrome and the purified cytochrome contains two moles haem C per mole polypeptide (Sone *et al.*, 2001). The gene *qcrC* sequence indicates that the subunit consists of two type-I cytochrome *c* domains with two haem C-binding motifs, $CXXCHX_nM$; thus, we named it 'cytochrome *cc*'. This gene and two others constitute a *qcrCAB* operon encoding a putative cytochrome *bc* complex. Similar *qcrCAB* operons are present in the genomes of other high-G+C Gram-positive bacteria, such as *Mycobacterium tuberculosis* (Cole *et al.*, 1998).

These findings prompted us to study the structural features of cytochrome *c* oxidase in this organism. Cytochrome *bd*-type oxidase and its structural genes have been isolated previously from *C. glutamicum* (Kusumoto *et al.*, 2000), and it has been determined that the enzyme is a menaquinol oxidase operating via an alternative electron-transfer pathway, as in other bacteria. In this work, we have isolated a cytochrome aa_3-type cytochrome *c* oxidase and have cloned its structural genes. In comparison with other cytochrome *c* oxidases, the enzyme has a long insertion containing both basic and acidic amino acid residues in the cytochrome *c*-binding domain of subunit II.

METHODS

Cell growth and membrane preparation. The cells of *C. glutamicum* KY9002 (ATCC 13032) were grown aerobically at 30 °C as described previously (Kusumoto *et al.*, 2000). Cells were harvested at the early stationary phase by centrifugation at 8000 *g* for 15 min and stored in a freezer. Cells of about 120 g wet weight were suspended in 200 ml 10 mM NaP_i buffer (pH 7·4) containing 0·5 % (w/v) NaCl and were disrupted by vigorous mixing with glass beads (diameter 0·5 mm, 350 g) in a cell-disrupting mixer Bead-Beater (Biospec) for 2 min, five times in ice water. Unbroken cells were removed by centrifugation at 8000 *g* for 10 min and then the supernatant was centrifuged at 100000 *g* for 60 min. The precipitate was resuspended in a buffer containing 100 mM NaCl and 50 mM KP_i at pH 6·5 and used as the membranes.

Enzyme preparation. The membranes were suspended at 5 or 10 mg protein ml^{-1} in a buffer containing 2 % (w/v) sodium cholate, 0·5 M NaCl and 10 mM NaP_i at pH 7·4, then sonicated for 2 min (five times). The membrane proteins sedimented at 100000 *g* for 30 min were resuspended in a buffer containing 2 % (w/v) *n*-decyl-D-glucoside (DG), 0·5 M NaCl, 50 mM KP_i, pH 6·5, then sonicated and centrifuged as described above. The extract was dialysed against 10 mM NaP_i (pH 7·4) and applied to a DEAE-Toyopearl column (1·4 × 10 cm). Absorbed proteins were eluted with a buffer containing 1 % DG, 10 mM NaP_i (pH 7·4) and increasing concentrations of NaCl. The peak fractions of cytochrome aa_3 at 200 mM NaCl were applied to a hydroxyapatite column (0·8 × 2 cm), then proteins were eluted with a 1 % DG solution containing increasing concentrations of NaP_i buffer. The cytochrome was mainly recovered at 200 mM NaP_i.

Enzymic activity. *N,N,N′,N′*-Tetramethyl-*p*-phenylenediamine (TMPD) oxidase activity was measured at 25 °C in the presence of 250 μM TMPD, 0·1 M NaCl, 1 mM EDTA and 50 mM NaP_i buffer at pH 6·5 by monitoring the increase in the A_{560} value, and was calculated by using a millimolar absorption coefficient (ε_{560}) of 10·5 mM^{-1} cm^{-1} (Sakamoto *et al.*, 1996). For cytochrome *c* oxidase activity, yeast or horse-heart cytochrome *c* was reduced with hydrosulfite and separated by a centrifuged column containing Sephadex G-50 (fine). The reaction was started by mixing cytochrome *c* with cytochrome aa_3 at final concentrations of 0·1 mM and 0·5 μM, respectively, in 20 mM NaP_i (pH 7·4). The amount of residual ferrocytochrome *c* was calculated from absorption spectra using a millimolar absorption coefficient (ε_{550}) of 19·1 mM^{-1} cm^{-1}. The oxidase activity of the membranes was measured using a Yellow Springs oxygen electrode (no. 4005) at 30 °C in a 2·3 ml semi-closed vessel containing a respiratory substrate in 20 mM KP_i buffer at pH 6·5, as described previously (Sakamoto *et al.*, 1997).

Cloning of the genes. Gene manipulations were carried out as described previously (Sakamoto *et al.*, 1999). To clone the gene for subunit II, a sense primer for PCR, 5′-GGYGAYTTCYTBCGNATGGG-3′ (crg1), and an antisense primer, 5′-GGACCGCASARYTCNGMRCA-3′ (crg2r), were designed on the basis of the N-terminal peptide sequence (GDFLRMG) and the highly conserved sequence of the Cu_A-ligating motif (C(A/S)ELCGP-), respectively. A PCR was performed using *C. glutamicum* chromosomal DNA as the template, and the resultant 0·8 kb product (AA1) was used as the probe. Chromosomal DNA was partially digested with *Sau*3AI, and the resultant fragments were ligated to *Bam*HI-digested pUC119. A positive clone (AA41) was obtained by colony hybridization; then, using this as the probe, two other clones (AA51 and AA61) were obtained. These clones contained whole *ctaC* and *ctaE* genes, which encode subunits II and III, but did not contain the gene for subunit I. To clone this gene, a set of PCR primers were prepared on the basis of the highly conserved sequences of subunit I of cytochrome *c* oxidases. A sense primer, 5′-TCATGGTNTGGGYNCAYCAY-3′ (uni1), was designed on the basis of a sequence (FMVW(A/V)HH-) between the seventh and eighth transmembrane segments and an antisense primer, 5′-ATAACRTWRTGRAARTGNGC-3′ (uni2r), was based on a sequence within the 10th segment (AHFH(Y/N)VI-). A fragment of about 0·3 kb (AA2) was solely produced and then used as a probe for Southern and colony hybridization to obtain a positive *Pst*I fragment (AA22) and a *Sph*I fragment (AA32), which make up the whole *ctaD* gene encoding subunit I.

Other analyses. Absorption spectra were recorded at room temperature, as described previously (Kusumoto *et al.*, 2000). The cytochrome aa_3 content was calculated from redox difference spectra by using a millimolar absorption coefficient (ε_{605}) of 21·0 mM^{-1} cm^{-1} (Sone & Yanagita, 1982). To estimate the molecular masses of haems, they were extracted with HCl/acetone, dried as described previously (Sakamoto *et al.*,

1997), dissolved in aqueous 30% acetonitrile solution, and then mixed at a 1:1 ratio with a 50% acetonitrile solution of 10 mg α-cyano-4-hydroxycinnamic acid ml^{-1} and 0·1% trifluoroacetic acid. The mixture was spotted onto a sample plate and analysed using a matrix-assisted laser desorption ionization (MALDI) mass spectrometer (Voyager LN-DE; PerSeptive Biosystems). MALDI mass spectrometry of proteins was performed using 2-(4-hydroxyphenylazo)benzoic acid as the matrix, as described by Ghaim *et al.* (1997). Reverse-phase chromatography of haems, the protein concentration assay, SDS-PAGE, and peptide sequence analysis were performed as described previously (Sakmoto *et al.*, 1997, 1999).

Materials. Cytochromes *c* from bovine heart and yeast were purchased from Sigma. DEAE-Toyopearl, Sephadex G-50 fine, 2-(4-hydroxyphenylazo) benzoic acid and hydroxylapatite were purchased from Tosoh, Pharmacia, Aldrich and Bio-Rad, respectively. MEGA 9, MEGA 10 and *n*-dodecyl β-D-maltoside were obtained from Dojin. Other reagents were of analytical grade.

RESULTS

Purification of cytochrome aa_3-type oxidase

Cells of *C. glutamicum* were harvested in the early stationary growth phase at an OD_{650} value of about 14. The redox difference spectrum of the cell membranes showed the presence of *a*-, *b*- and *c*-type cytochromes (Fig. 1a, inset). The presence of the *a*-type cytochrome suggests that the main respiratory oxidase is cytochrome aa_3. The oxidase activity in the membrane preparations in the presence of 0·2 mM NADH and TMPD as the respiratory substrate was 560 and 120 ng-atomO min^{-1} (mg protein)$^{-1}$, respectively. The activity was as low as 12 and 10 ng-atomO min^{-1} (mg protein)$^{-1}$ with 10 μM yeast and horse-heart cytochromes *c* as the substrates, respectively. The solubility of the oxidase was tested in the following detergents: sodium cholate, Triton X-100, sucrose monolaurate, DG, *n*-dodecyl β-D-maltoside, and a 1:1 mixture of *n*-nonanoyl *N*-methylglucamide and *n*-decanoyl *N*-methylglucamide (MEGA 9+MEGA 10). Sodium cholate was ineffective at solubilizing the *a*-type cytochrome but was effective at removing the peripheral proteins. About the same amount of enzyme activity was retained immediately after solubilization in Triton X-100, DG, *n*-dodecyl β-D-maltoside, and MEGA 9+MEGA 10, and the highest activity remaining 3 d later was in DG and in *n*-dodecyl β-D-maltoside. DG was chosen as the detergent for solubilizing the oxidase for ease of availability. This enzyme stability in DG was not affected by the presence of either 15% glycerol, 1% asolectin or a combination of peptidase inhibitors (1 mM benzamidine hydrochloride, 0·2 mM PMSF and 1 mM EDTA) (data not shown). After solubilization with DG, the proteins were fractionated by DEAE-Toyopearl anion-exchange chromatography and then by hydroxyapatite chromatography (Table 1). The turnover number for TMPD oxidase activity dropped about 10-fold with solubilization. The isolated cytochrome catalysed the oxidation of TMPD, horse-heart cytochrome *c* and yeast cytochrome *c*, with turnover numbers of 0·61, 0·23 and 2·54 s^{-1}, respectively, indicating that the *a*-type cytochrome is a cytochrome *c* oxidase, though these values are much lower than those of other cytochrome *c* oxidases. The activity was not enhanced by the addition of phospholipid extracted from the cells. The cytochrome did not catalyse the oxidation of menaquinol-1, -2, -3, dimethylnaphthoquinol, menadiol, ubiquinol-1 or ubiquinol-2 (data not shown), in contrast to the cytochrome *bd*-type quinol oxidase recently isolated from this organism (Kusumoto *et al.* 2000). In addition, it did not catalyse the oxidation of azurin from *Pseudomonas aeruginosa*.

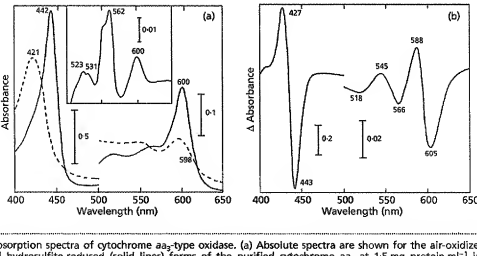

Fig. 1. Absorption spectra of cytochrome aa_3-type oxidase. (a) Absolute spectra are shown for the air-oxidized (broken lines) and hydrosulfite-reduced (solid lines) forms of the purified cytochrome aa_3 at 1·5 mg protein ml^{-1} in 1% DG, 200 mM NaP_i (pH 7·4). Inset: redox difference spectrum of a membrane preparation at 5 mg protein ml^{-1} in 5% Triton X-100 and 50 mM sodium phosphate (pH 7·4). (b) Difference spectrum of the CO-bound reduced *minus* the reduced form of the purified cytochrome aa_3. The content was the same as for (a).

Table 1. Purification of aa_3-type cytochrome *c* oxidase

Purification steps	Total protein (mg)	Cytochrome aa_3		TMPD oxidase activity		
		Total (nmol)	Specific (nmol mg^{-1})	Total (units)	Specific (units mg^{-1})	TN* (s^{-1})
Membranes	662	41·0	0·0619	26·7	0·0403	10·8
Cholate-washed	583	42·7	0·0732	19·4	0·0333	7·51
DG-extracted	47·8	44·2	0·925	1·82	0·0381	0·68
DEAE-Toyopearl	6·19	13·2	2·13	1·51	0·244	1·91
Purified aa_3	0·341	2·76	8·09	0·101	0·296	0·61

* Moles TMPD oxidized per mole cytochrome aa_3 per second.

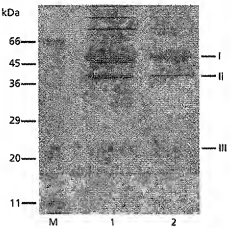

Fig. 2. SDS-PAGE analysis of cytochrome aa_3-type oxidase. Electrophoresis was performed using a 13·5% (w/v) acrylamide gel as described in Methods. Lane M, molecular mass standards; lane 2, purified oxidase (8 μg); lane 3, the same sample (3 μg). The roman numbers on the right indicate the positions of the three subunits.

Chromophore and subunit composition

The reduced form of the purified cytochrome showed the α- and γ-peaks at 600 and 442 nm, respectively (Fig. 1a). The difference spectrum of the CO-reduced *minus* reduced forms demonstrates that the wavelength shift of the absorption peaks is due to haem A (Fig. 1b). These data clearly indicate that the cytochrome is of the aa_3 type. The haem A was extracted from the oxidase and analysed with reverse-phase chromatography and MALDI mass spectrometry. The haem was eluted from the reverse-phase column at a higher acetonitrile concentration than that of the control haem A extracted from thermophilic *Bacillus* cells (Sakamoto *et al.*, 1997), suggesting that it is more hydrophobic than the usual type of haem A (data not shown). The molecular mass of the haem estimated by mass spectrometry was 920·09 Da, which is higher than that for normal haem A (852·85 Da). The difference can be explained if it is assumed that the haem A of this oxidase has the geranylgeranyl side-chain ($C_{20}H_{39}$-) instead of the farnesyl group ($C_{15}H_{29}$-), as demonstrated for the haem As in archaeal quinol oxidase (Lübben & Morand, 1994). Haem B extracted from the membranes of *C. glutamicum* showed a molecular mass of 616·20 Da, which indicates that it is protohaem IX, the usual type of haem B (mol. mass 616·50 Da). SDS-PAGE analysis indicated that the final sample contained three main polypeptides, although the third band was relatively faint and was clearly visible only when the sample was overloaded (Fig. 2). The molecular masses estimated by using Ferguson plots were 64·3, 40·5 and 21·0 kDa, respectively. The molecular mass of the whole enzyme estimated from gel filtration was 160 kDa, which is compatible with the assumption that the enzyme is a heterotrimer bound by detergent micelles. The N-terminal sequence of subunit II was determined to be XEVAPPGGVLGDFLRMGWPDGI- by automated Edman degradation, whereas no appreciable amounts of PTH-amino acids were obtained from subunit I or subunit III.

Genes for the three subunits

The *ctaC* gene for subunit II was isolated by using a probe prepared on the basis of the N-terminal peptide sequence of the subunit (Fig. 3a). The *ctaE* gene for subunit III was 1031 bp downstream of *ctaC*, whereas the gene for subunit I was not found in the vicinity. Therefore a new set of PCR primers was designed on the basis of highly conserved sequences in subunit I of the haem–copper oxidase superfamily. Thus, the whole *ctaD* gene for subunit I was obtained (Fig. 3b). The total numbers of amino acid residues deduced from the genes are 584, 359 and 205, and the molecular masses are 65032, 39518 and 22442 Da, respectively. The molecular masses estimated by MALDI mass spectrometry were 64955·8 ± 164·1, 37314·7 ± 83·2 and 22400·7 ± 27·9 Da (mean ± SEM, $n = 6$), respectively. A comparison of these values indicated that subunit II, but not subunit I or subunit III, was significantly modified after translation (see below). The haem–copper oxidase family can be classified into the following three subgroups on the basis of the subunit composition and the primary structure: the SoxM group includes mito-

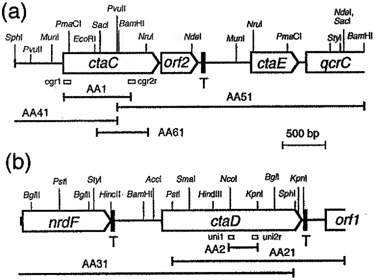

Fig. 3. Physical map of the genes for the three subunits of cytochrome aa_3-type oxidase. Genes are indicated by open arrows. Solid rectangles (T), putative terminators with a palindromic sequence; open rectangles (cgr1, cgr2r, uni1, uni2r), PCR primers. PCR products (AA1, AA2) were used as probes to obtain the other clones (AA41, AA51, AA61, AA21, AA31).

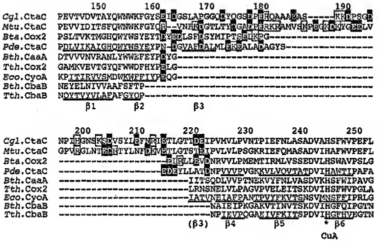

Fig. 4. Alignment of subunit II of several haem–copper oxidases. CtaC of *C. glutamicum* (*Cgl.*) was compared with eight counterparts by using CLUSTAL W (Thompson *et al.*, 1994) followed by manual ajustment. The DDBJ/EMBL/GenBank accession numbers are as follows: *M. tuberculosis* (*Mtu*) CtaC, Z70283; bovine cardiac (*Bta*), P00404; *P. denitrificans* (*Pde*) CtaC, P08306; *B. thermodenitrificans* (*Bth*) CaaA, D70843; *T. thermophilus* (*Tth*) CoxB, M59180; *E. coli* (*Eco*) CyoA, J05492; *Bth* CbaB, AB008757; *Tth* CbaB, L09121. Solid underlines, β-strands identified in the crystal structures; open boxes, basic residues between β2 and β4 strands; solid boxes, acidic residues in the same regions.

chondrial oxidases, the SoxB group includes some oxidases from extremophiles, and the FixN group consists of cytochrome cbb_3 from microaerobic and aerobic proteobacteria (Castresana & Saraste, 1995). Sequence comparison shows that the new oxidase belongs to the SoxM-type subfamily and is most similar to oxidases in other high-G+C Gram-positive bacteria, such as *M. tuberculosis* (Cole *et al.*, 1998).

Structural features of subunit II

Comparison of the subunit II sequence obtained by Edman degradation (see above) with that deduced from the nucleotide sequence indicates that the N-terminal signal peptide of the subunit is post-translationally cleaved between Gly28 and Cys29. The PTH-derivative of the first residue was not detected, while those of the following residues were clearly identified, suggesting that the Cys residue is modified but not *N*-acetylated. The cleaved site, -M-A-G-C-, fits the signal-sequence motif of lipoproteins, -L/M-A/S-G-C-, and is similar to those of the subunits II of other oxidases (Ishizuka *et al.*, 1990; Quirk *et al.*, 1993; Ma *et al.*, 1997; Bengtsson *et al.*, 1999). It was empirically demonstrated that the thiol group is diacylglycerated in the caa_3-type cytochrome *c* oxidase from *B. subtilis* and the bo_3-type quinol oxidase from *Escherichia coli*. If it is assumed that the N-terminal 28 residues of *C. glutamicum* subunit II are cleaved off and that the new N-terminal cysteine residue is distearoylglycerated, the calculated molecular mass is 37275 Da, which fits reasonably with the value estimated by MALDI mass spectrometry (see above). The atomic structures of the whole enzyme have been solved for cytochromes aa_3 of *Paracoccus denitrificans* (Iwata *et al.*, 1995) and of bovine mitochondria (Tsukihara *et al.*, 1996), cytochrome *bo* of *E. coli* (Abramson *et al.*, 2000) and cytochrome ba_3 of *Thermus thermophilus* (Soulimane *et al.*, 2000). The C-terminal extrinsic

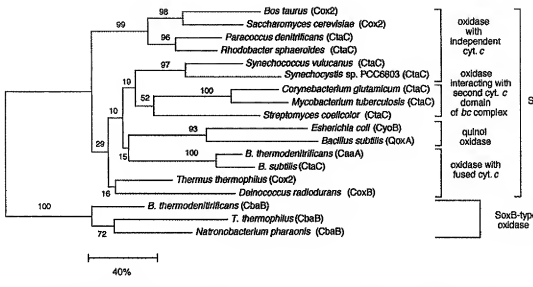

Fig. 5. Phylogenetic tree for subunit II of haem–copper oxidases. The tree was constructed on the basis of sequences between the transmembrane helix II and the 10th β-strand (β10) in the extrinsic domain of the subunit with CLUSTAL W, as described for Fig. 4. Additional sequence data were obtained from the DDBJ/EMBL/GenBank databases using the following accession numbers: *Saccharomyces cerevisiae* Cox2, V00685; *Rhodobacter sphaeroides* CtaC, M57680; *Synechococcus vulcanus* CtaC, P98054; *Synechocystis* sp. PCC6803 CtaC, D90905; *B. subtilis* CtaC, X54140; *Streptomyces coelicolor* Cox2, AL049497; *Deinococcus radiodurans* CoxB, AE002091; *Natronobacterium pharaonis* CbaB, Y10500. The numerals represent bootstrap confidence levels from 1000 bootstrap samples for the groupings. The scale bar represents a distance of 40%.

domain of subunit II of these oxidases contains 10 β-strands. Cytochrome aa_3 from *C. glutamicum* contains the Cu_A-binding motif ($HX_nCXEXCGX_2HX_2M$) in the same domain as the other SoxM- and SoxB-type cytochrome *c* oxidases.

The most distinguishable structural feature is the extra cluster of charged amino acid residues between the $\beta2$ and $\beta4$ strands (Fig. 4). The length of this region varies among subgroups of the haem–copper oxidases. The first group consists of SoxB-type cytochrome *c* oxidases, which have the shortest $\beta2$–$\beta4$ spans. The second group includes the caa_3-type cytochrome *c* oxidases and proteobacterial ubiquinol oxidases, in which this region is about 10 residues longer than that in the SoxB-type group. The third group is the largest, including all of the aa_3-type cytochrome *c* oxidases from mitochondria and proteobacteria. These contain $\beta2$–$\beta4$ regions, rich in acidic residues, approximately 20 residues longer than those in the second group. Finally, as shown in this study, *C. glutamicum* aa_3-type oxidase, together with homologues from other high-G+C Gram-positive bacteria, contains the longest $\beta2$–$\beta4$ span (being about 30 residues longer than that in the third group), rich in both acidic and basic amino acids. The last three groups are all SoxM-type oxidases (Fig. 5).

DISCUSSION

C. glutamicum cytochrome aa_3 is a cytochrome *c* oxidase, as indicated by its enzymic activity and the presence of the Cu_A-binding motif in subunit II (Fig. 4). In spite of these clear indications, *C. glutamicum* cells do not contain small cytochrome *c* which could serve as the intrinsic substrate. The only *c*-type cytochrome identified in this organism is the cytochrome *cc* subunit of the *bc* complex encoded by the *qcrCAB* operon (Sone *et al.*, 2001). The presence of a homologous gene and the absence of a gene for small cytochrome *c* are confirmed in the whole genome of *M. tuberculosis*, a high-G+C Gram-positive bacterium related to *C. glutamicum* (Cole *et al.*, 1998). Cytochrome *cc* contains two type-I cytochrome *c* domains. These findings suggest that one of the two domains plays a role equivalent to that of the cytochrome c_1 of the bc_1 complex, and the other a role comparable to that of small cytochrome *c*. In other words, the cytochrome *cc*-containing *bc* complex might interact with the aa_3 oxidase in the absence of an electron carrier. In this context, one likely reason for the decline in TMPD oxidase activity upon solubilization (Table 1) is that the *bc* complex and the aa_3 oxidase are associated in the intact membrane and dissociated upon solubilization, since TMPD is a more effective donor when cytochrome *c* is bound to subunit II.

Genes for di-haem C proteins in *bc* complexes are also found in the genomes of the ε-proteobacteria, such as *Helicobacter pylori* (Tomb *et al.*, 1997), and in the photoautotrophic low-G+C Gram-positive *Heliobacillus mobilis* (Xiong *et al.*, 1998). However, the only respiratory oxidase in the former organism is cytochrome cbb_3 (lacking a subunit-II homologue), and the *bc* complex in the latter bacterium is encoded in a major photosynthesis gene cluster. The high-G+C Gram-

positive bacteria have both a cytochrome *cc*-containing *bc* complex and a subunit-II-containing terminal oxidase (Figs 4 and 5). The subunit II of these oxidases commonly contains an extra charged amino acid cluster in its cytochrome *c*-binding domain. These findings suggest that the inserted cluster may play a crucial role in the direct interaction and/or electron transfer between the *bc* complex and the terminal oxidase in the new type of respiratory chain.

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